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Reversible and irreversible effects of retinol upon the phenotypic properties of embryonal carcinoma cells

MICHAEL I. SHERMAN, MARTIN A. EGLITIS* AND RICHARD THOMAS

Roche Institute of Molecular Biology, Roche Research Centre, Nutley, New Jersey 07028, USA

SUMMARY

We have generated an embryonal carcinoma cell line, NR1, which is not growth-inhibited in response to retinol. Although the retinol-treated cells undergo morphological change, show reduced levels of the surface antigen SSEA-1 and possess increased surface reactivity with anti-fibronectin serum, the extent of phenotypic change of NR1 cells in response to retinol is not so great as that following treatment with retinoic acid. Furthermore, unlike cells from lines such as F9, the retinol-promoted morphological alterations appear to be reversible. The increased adherence of NR1 cells to glass coverslips in the presence of retinol suggests that stronger interaction with the substratum is responsible for the observed alteration in appearance of the cells. It is possible that NR1 cells exposed to retinol progress no further than a reversible, early stage of differentiation. Alternatively, retinol-treated cells might express a group of markers normally associated with, or accompanying, an irreversible, differentiated phenotype even though the cells are not, in fact, undergoing differentiation.

INTRODUCTION

Since embryonal carcinoma (EC) cells are considered to be pluripotent, they can theoretically differentiate into a spectrum of cell types. Accordingly, when one assesses the state of differentiation of cells in EC cultures, it would seem prudent to use several general discriminatory markers. Among these are alterations in morphology, elevated levels of secretion of plasminogen activator (e.g. Sherman *et al.* 1976), cell-surface deposition of intercellular matrix proteins such as fibronectin and laminin (e.g. Zetter & Martin, 1978; Wolfe, Mautner, Hogan & Tilly, 1979; Knowles *et al.* 1980), appearance of cytoskeletal elements such as keratin (e.g. Jones-Villeneuve, McBurney, Rogers & Kalnins, 1982), and disappearance of EC-specific surface antigens including SSEA-1 (Solter & Knowles, 1978). In studies with several EC lines and differentiation-defective EC cell mutants derived from them (McCue, Matthaei, Taketo & Sherman, 1983), we have shown that these parameters are collectively useful in attempts to evaluate differentiation, even though they do not all invariably change when differentiation

* Present address: Laboratory of Molecular Hematology, National Institutes of Health, Bethesda, Maryland 20205, USA.

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is induced. According to classical concepts of differentiation, it is also to be expected that such alterations in phenotype are permanent, i.e. they should not be reversed after removal of the inducer (see Sherman, 1986*a*).

Among known inducers of differentiation of EC cells, retinoids, especially retinoic acid, are particularly active (reviewed recently by Jetten, 1986). Retinol is less potent than retinoic acid in inducing differentiation of EC cells: quantitative differences have been evidenced among various lines (Eglitis & Sherman, 1983) and even within a given line, F9, when studied by different laboratories (Strickland & Mahdavi, 1978; Eglitis & Sherman, 1983; Jetten & DeLuca, 1983). Furthermore, one EC line, PCC4.aza1R, differentiates in response to retinoic acid but fails to do so when treated with retinol at non-toxic concentrations (Jetten & Jetten, 1979; Eglitis & Sherman, 1983). Nulli-SCC1 is an EC line which appears to respond, albeit poorly, in response to retinol (Eglitis & Sherman, 1983). We have derived from Nulli-SCC1 a clonal line, which we have called NR1. As described below, this cell line responds in an uncharacteristic manner to retinol. The behaviour of these cells raises some fundamental questions about the criteria used to assess differentiation of EC cells.

MATERIALS AND METHODS

Cell lines and culture conditions used

The Nulli-SCC1 line was described initially by Martin & Evans (1975) and was obtained from Dr Gail Martin. Culture of these cells in serum- and antibiotic-supplemented Dulbecco's modified Eagle's medium in gelatin-coated dishes is described by McCue *et al.* (1983). The NR1 line was derived from Nulli-SCC1 cells after treatment with *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine as described by Schindler *et al.* (1981). Retinol ($0{\cdot}25\,\mu g\,ml^{-1}$, or $8{\cdot}7\times10^{-7}$ M) was added 24 h after cells were treated with mutagen and seeded at clonal density. NR1 cultures were maintained in the same way as Nulli-SCC1 cultures. The origin and maintenance of F9 EC cells have been described elsewhere (Eglitis & Sherman, 1983). For growth curves, cells were plated in gelatin-coated 6 cm culture dishes at densities of $1–2\times10^5$ cells.

Retinoid treatments

When cells were treated with all-*trans*-retinol ($8{\cdot}7\times10^{-7}$ M) or all-*trans*-retinoic acid (10^{-6} M) the retinoids were added in ethanol such that the concentration of vehicle did not exceed 0·1%. It has been shown previously (Jetten & Jetten, 1979) that this concentration of vehicle has no detectable effect on EC cells. Retinoids were stored as 10^{-2} M (retinoic acid) or $8{\cdot}7\times10^{-3}$ M (retinol) stock solutions and kept for up to two weeks at −70°C. HPLC analyses have indicated that the retinoids retain their integrity under these conditions (M. L. Gubler, personal communication). Cultures were maintained in a room equipped with amber lights.

Assessment of differentiation

The proportion of cells with non-EC morphology was estimated by examining several fields in two or more dishes with a phase-contrast microscope. (Cells with a typical EC morphology are small and rounded with indistinct boundaries and high nuclear/cytoplasmic ratios; see, e.g. Figs 3A, 4A.) For measurements of secretion of plasminogen activator, culture media were assayed for plasminogen-dependent degradation of ^{125}I-fibrin (described in detail by Eglitis & Sherman, 1983). Surface-associated fibronectin was detected by immunofluorescence with rabbit anti-human fibronectin (BRL, Bethesda, MD: diluted 1:5) and loss of SSEA-1 antigen by immunofluorescence with monoclonal antibody (diluted 1:100 or 1:200) kindly supplied by Drs D.

Solter and B. Knowles (see Solter & Knowles, 1978). Cells were also challenged with rabbit antisera against laminin and the cytoskeletal component keratin. These antisera were obtained from Drs R. Oshima and H. Sun and diluted at 1:5 and 1:30, respectively. In all instances, fluorescein-conjugated second antibody (diluted 1:20) was used. Appropriate non-immune sera were used on control coverslips. The immunofluorescence procedure used was the same as that described by Eglitis & Sherman (1983) except that in the present study, cells were cultured on glass coverslips and fixed for 6 min with ice-cold acetone (for SSEA-1), methanol (for fibronectin or laminin) or with methanol for 6 min followed by acetone for 2 min (for keratin).

Tumour analyses

Cells for tumour analyses were cultured for the indicated time periods in the absence or continuous presence of retinol at $8{\cdot}7\times10^{-7}$ M, with passage as the cells approached confluence. Cells were harvested by trypsinization, washed and resuspended in phosphate-buffered saline. After counting, 5×10^5, 10^6 or 2×10^6 cells were each injected s.c. into the left flank of strain 129 mice. Mice injected with control or retinol-treated cells at each inoculation level were matched for age and sex. Mice were inspected at least three times per week and the earliest time of appearance of a palpable tumour was noted.

RESULTS

Generation of the NR1 line

After Nulli-SCC1 cells were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and exposed continuously to retinol at $8{\cdot}7\times10^{-7}$ M, a clonal line, NR1, was isolated. Unlike the parental cells, NR1 cells were not growth-inhibited by retinol, as reflected by typical growth curves illustrated in Fig. 1A,B. However, the density of NR1 cells at confluence was lower in the presence of retinol than in its absence (Fig. 1C).

Plasminogen activation analyses

Table 1 illustrates that Nulli-SCC1 cells secreted 30-fold increased levels of plasminogen activator following 8 days of exposure to retinol, with only a modest increase after 4 days, in confirmation of an earlier report (Eglitis & Sherman, 1983). Also as demonstrated previously (McCue *et al.* 1983), the same cells responded readily to a similar dosage of retinoic acid within 4 days. The response of NR1 cells to retinol was much less marked than that of Nulli-SCC1 cells: levels of plasminogen activator secretion were elevated only five-fold after 8 days (Table 1). On the other hand, secretion levels increased more than 50-fold when NR1 cells were exposed to retinoic acid for 4 days.

In a separate experiment involving long-term treatment with retinol, Nulli-SCC1 cells secreted maximal amounts of plasminogen activator after 14 days of treatment, with the level falling dramatically by 28 days (Fig. 2A). NR1 cells consistently failed to secrete markedly elevated levels of plasminogen activator (Fig. 2B).

Morphological analyses

Fig. 2A reveals that although Nulli-SCC1 cells produced a large increase in plasminogen activator secretion in response to retinol, the estimated percentage of

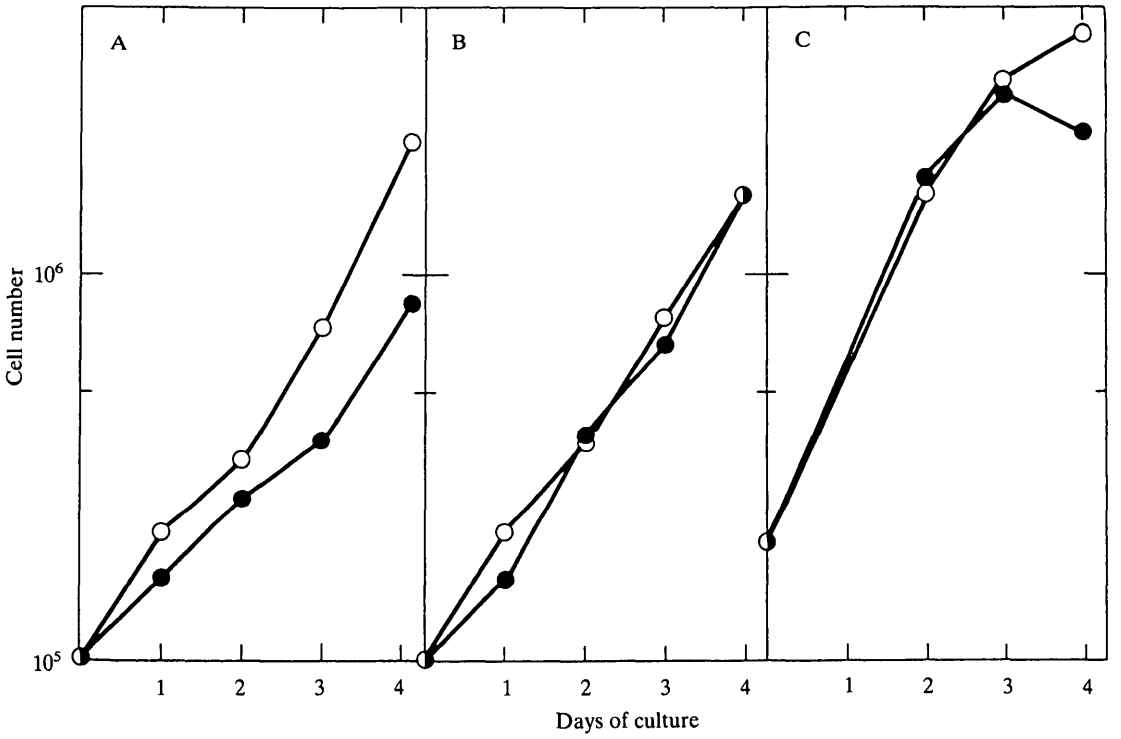

Fig. 1. Effects of retinol on the growth of Nulli-SCC1 and NR1 embryonal carcinoma cells. Nulli-SCC1 (A) or NR1 (B,C) cells were seeded at 10^5 (A,B) or 2×10^5 (C) in 60 mm diameter culture dishes in the absence (○) or presence (●) of $8{\cdot}7\times10^{-7}$ M-retinol. Cell numbers were determined at the times indicated. Each point represents the average of triplicate determinations. Note that the scale on the ordinate is a log scale.

Table 1. *Plasminogen activator secretion by Nulli-SCC1 and NR1 cells in response to retinoids*

Cell line	Treatment†	Plasminogen activator secretion* 4 days	8 days
Nulli-SCC1	none	6·1	5·0
	retinol	20·8	153·1
	retinoic acid	266·5	ND‡
NR1	none	3·0	4·6
	retinol	8·6	23·9
	retinoic acid	197·1	ND‡

* Data are averaged from duplicate determinations in two to four independent assays and are expressed as c.p.m. [^{125}I]fibrin solubilized/10^5 cells.

† Retinoid treatment was continuous for the times indicated at $8{\cdot}7\times10^{-7}$ M (retinol) or 10^{-6} M (retinoic acid).

‡ Not determined.

cells showing obvious morphological alteration remained relatively low throughout the test period. By contrast, a large percentage of NR1 cells underwent morphological change despite the fact that there was little elevation of secretion of plasminogen activator (Fig. 2B). Figs 3–5 document the change in appearance of NR1 cells in response to retinol. Figs 3A and 4A illustrate that in control cultures the cells have a typical EC morphology. After only 2 days of exposure to retinol, although the individual cells did not look different, a subtle alteration appeared in the contours of the cell clusters: instead of being relatively smooth and rounded, they became more irregular and pointed (cf. Fig. 3A,B). By 4 days of treatment, some of the cells individually became more stellate. This property continued until, by 9 days, most of the cells in the culture possessed an irregular appearance (Fig. 4B); at this time the cells were also less likely to form tight clusters in which cell boundaries were obscured, as is typical of EC cells (e.g. Fig. 4A). At higher densities, retinol-treated NR1 cells tended to stream, as do fibroblasts (Fig. 5A). The cells retained their atypical appearance for at least 28 days of treatment with retinol (Fig. 5C).

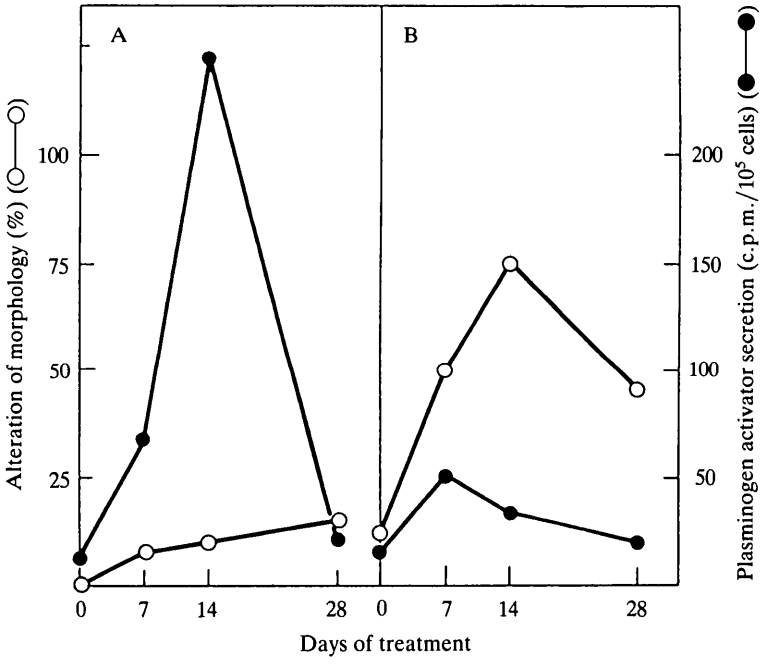

Fig. 2. Effects of retinol on morphology of, and plasminogen activator secretion by, Nulli-SCC1 and NR1 cells. Cells were cultured in the presence of retinol at $8{\cdot}7\times10^{-7}$ M with thrice weekly medium changes and subculture as the cells approached confluence. After 6, 13 and 27 days, fresh medium was added which contained plasminogen-depleted serum. 24 h later estimates were made of the proportion of cells in the cultures which did not possess a typical EC morphology (○), cells were counted, media were assayed for plasminogen activator content and the values, averaged from duplicate determinations, were expressed on a per cell basis (●). (A) Nulli-SCC1 cells; (B) NR1 cells.

When retinol was removed from NR1 cultures previously treated for up to two weeks with the retinoid, there was a rapid and remarkable reversal of morphology: within 2 days, most of the cell clusters became smooth and non-streaming once again (Figs 4C, 5B); individually, the cells lost their irregular appearance and became tightly adherent. It is only after cells had been treated with retinol for

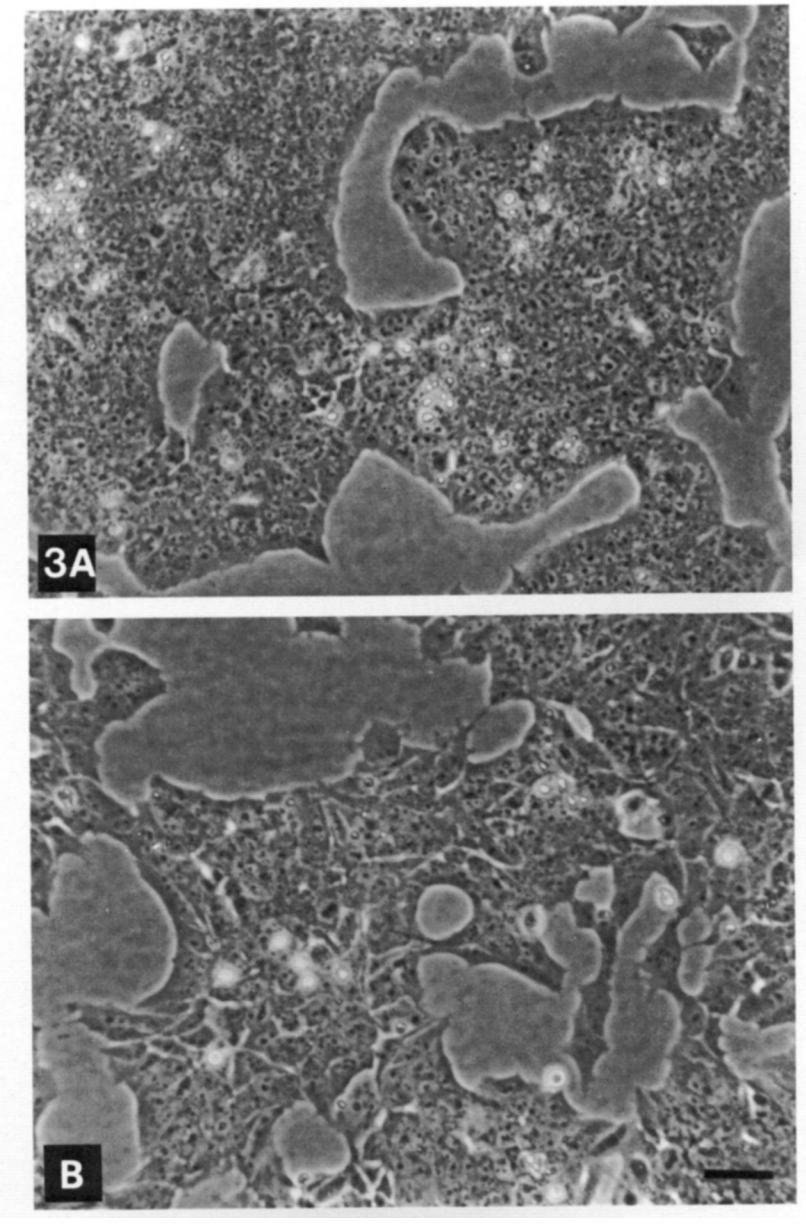

Fig. 3. Gross morphology of NR1 cells in response to retinol. (A) Phase-contrast photomicrograph of control NR1 cells; (B) NR1 cells exposed to $8{\cdot}7\times10^{-7}$ M-retinol for 2 days. Scale marker (in B) equals 50 μm.

extended time periods that they failed to undergo this morphological reversal within two days of withdrawal of retinol (e.g. Fig. 5D).

The reversibility of the morphological change of NR1 cells in response to retinol appears to be atypical. It was not, for example, observed when the cells were

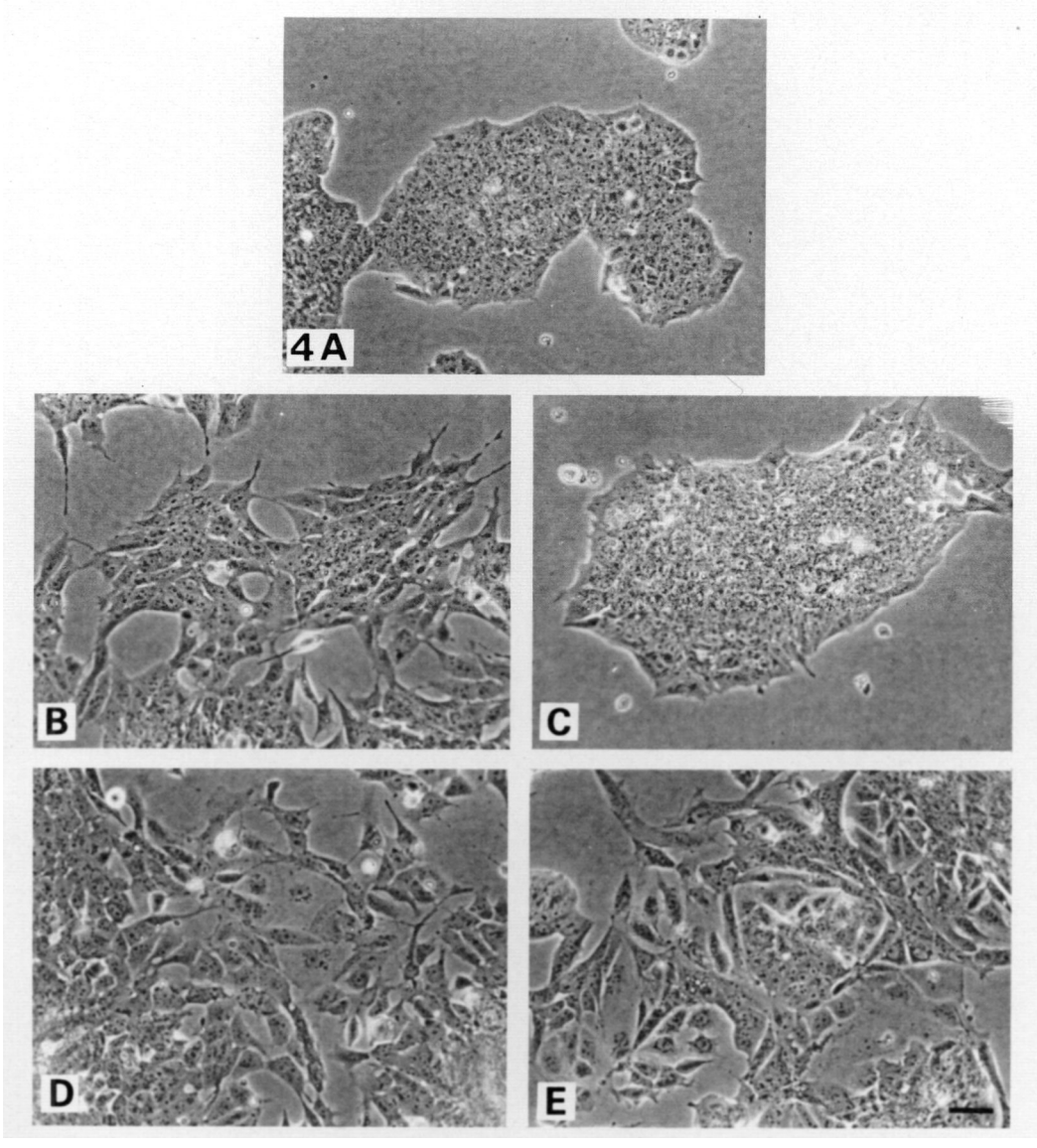

Fig. 4. Morphological responses of NR1 cells to retinol and retinoic acid. (A) Phase-contrast micrograph of control NR1 cells; (B) cells were exposed to $8{\cdot}7\times10^{-7}$ M-retinol for 9 days; (C) cells were exposed to $8{\cdot}7\times10^{-7}$ M-retinol for 7 days, washed, and cultured in medium free of exogenous retinol for 2 days; (D) cells were exposed to 10^{-6} M-retinoic acid for 9 days; (E) cells were exposed to 10^{-6} M-retinoic acid for 7 days, washed, and cultured in retinoic-acid-free medium for 2 days. Scale marker (in E) equals 50 μm.

exposed to retinoic acid; in this case, the stellate, irregular appearance of the cells (not markedly different from that observed when the cells were treated with retinol; cf. Fig. 4B,D) failed to reverse when that effector was removed (Fig. 4E).

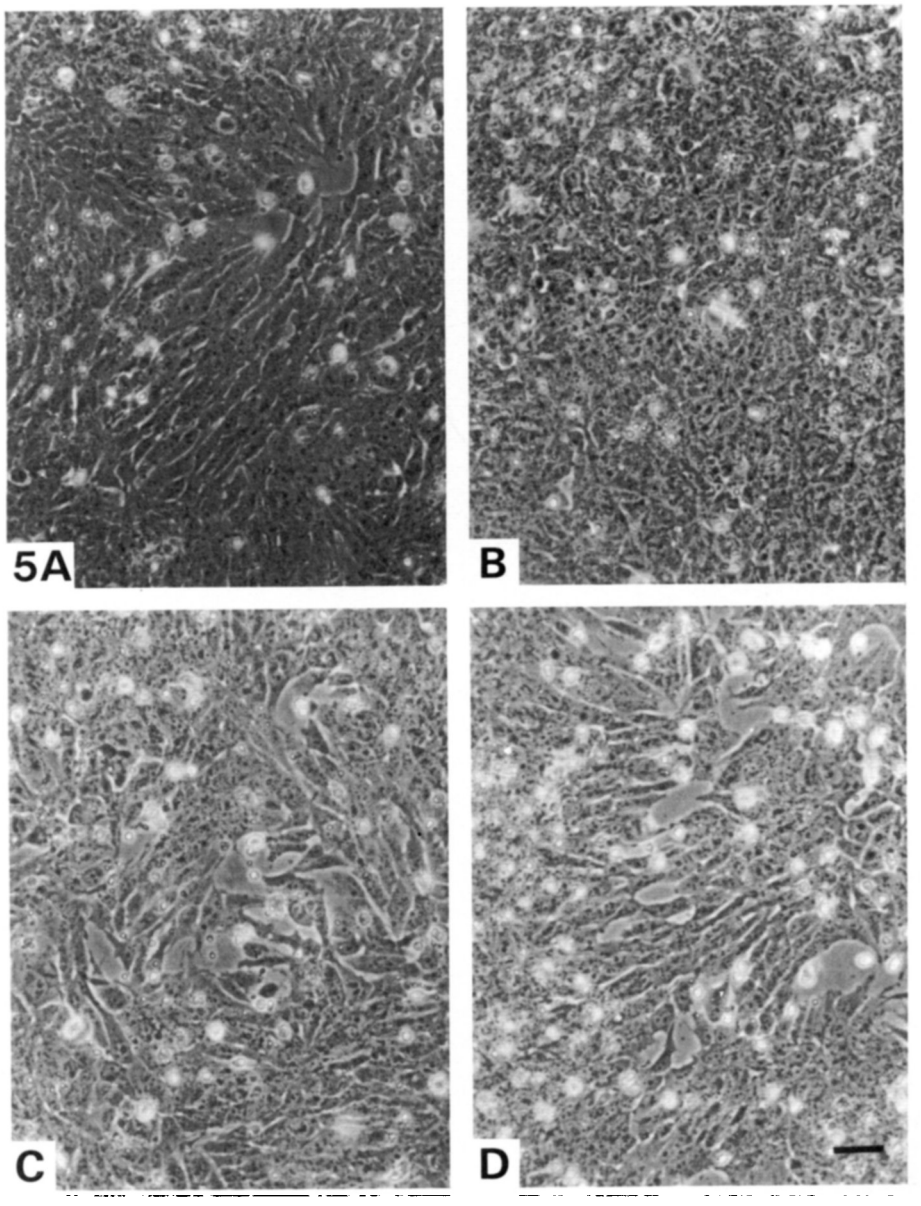

Fig. 5. Effects of long-term treatments with retinol on morphology of NR1 cells. (A) Phase-contrast micrograph of NR1 cells treated with $8{\cdot}7\times10^{-7}$ M-retinol for 14 days (medium was changed three times weekly and cells were passaged when they approached confluence); (B) cells were treated with $8{\cdot}7\times10^{-7}$ M-retinol for 12 days, and cultured for 2 days in medium lacking exogenous retinol; (C) as in A, except that exposure time to retinol was 28 days; (D) as in B, except that exposure time to retinol was 26 days. Scale marker (in D) equals 50 μm.

Fig. 6 illustrates that those cells in Nulli-SCC1 cultures which underwent morphological change in response to retinol retained their altered morphology after the retinol had been removed. Similarly, F9 cells, which we have reported to

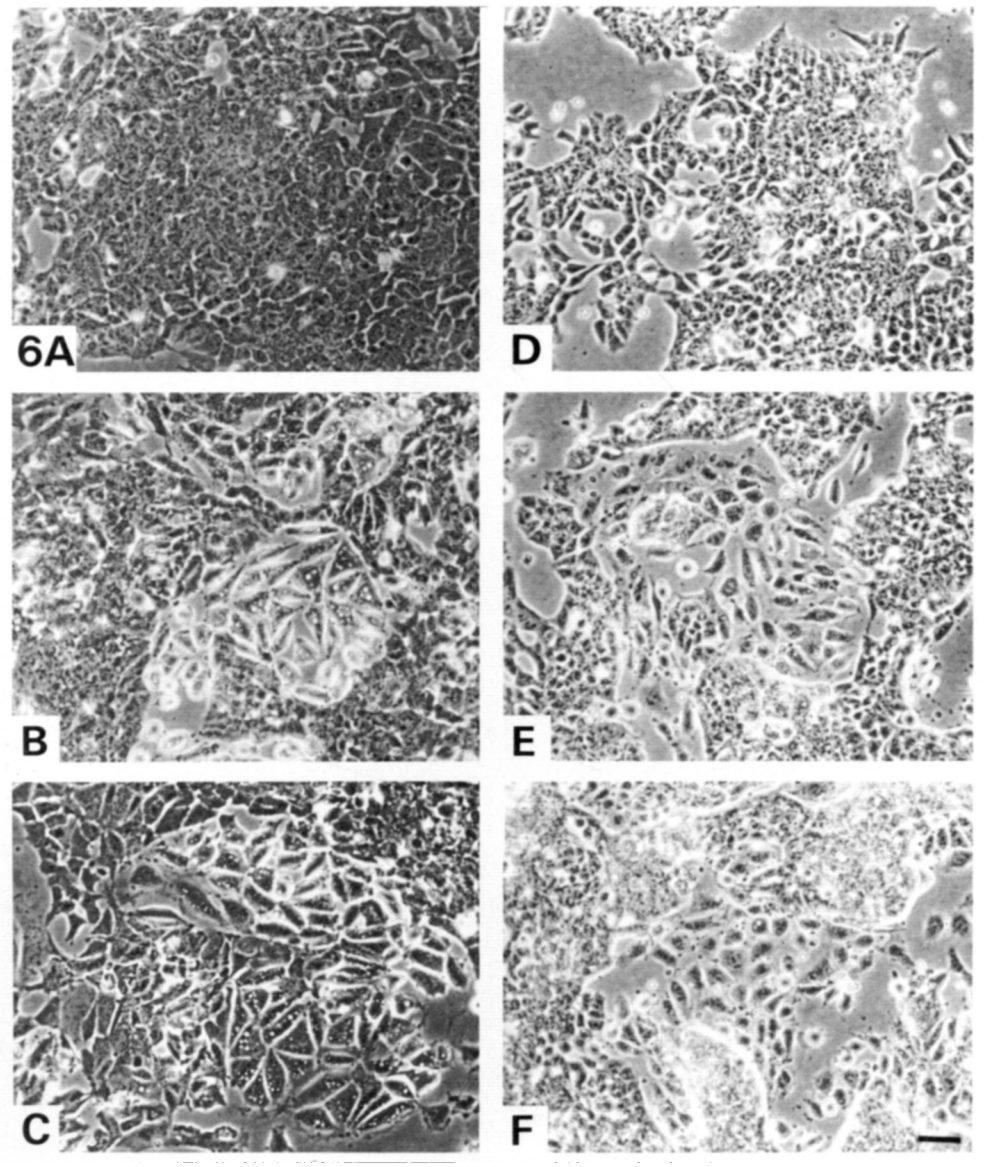

Fig. 6. Effects of retinol on the morphology of Nulli-SCC1 and F9 cells. Medium was changed three times weekly and cells were passaged as they approached confluence. (A) Phase-contrast micrograph of untreated Nulli-SCC1 cells; (B) Nulli-SCC1 cells were cultured in the presence of $8{\cdot}7\times10^{-6}$ M-retinol for 9 days; (C) Nulli-SCC1 cells were cultured for 7 days with $8{\cdot}7\times10^{-7}$ M-retinol, washed and then cultured for 2 days in medium lacking exogenous retinol; (D) untreated F9 cells; (E) F9 cells were cultured in the presence of $3{\cdot}5\times10^{-7}$ M-retinol for 9 days; (F) F9 cells were cultured for 7 days with $3{\cdot}5\times10^{-7}$ M-retinol, washed and then cultured for 2 days in medium lacking exogenous retinol. Scale marker in (C) equals 50 μm.

undergo extensive alteration in morphology (as well as other parameters) in response to retinol (Eglitis & Sherman, 1983), did not readily reverse their appearance following removal of that retinoid. The same irreversibility in appearance has been found (data not shown) for another cell line, OC15-S1, that differentiates readily in response to retinol (Eglitis & Sherman, 1983).

Tumour analyses

NR1 cells can be maintained in a proliferative state in the presence of retinol for long periods of time. To determine whether they possessed the tumourigenic capacity of EC cells (as opposed to differentiated derivatives which tend to be benign), NR1 cells maintained in the presence of retinol for 120 days were injected s.c. into six mice (twice each at 5×10^5, 10^6 and 2×10^6 cells). The average time for tumour appearance in the six mice was 15·7 days *versus* 15·2 days for tumours derived from untreated NR1 cells. In similar experiments, tumour incidence of Nulli-SCC1 cells was also 100% after exposure to retinol for 37 days, though the average time for appearance of the tumours, 18·0 days, was twice as long as that observed from control cells (9·0 days).

Immunofluorescence analyses

We tested NR1 and Nulli-SCC1 cells for the presence of surface-associated SSEA-1 antigen (Table 2). In untreated cultures, a higher proportion of NR1 than parental Nulli-SCC1 cells contained detectable levels of the antigen. With both

Table 2. *Immunofluorescence analysis of SSEA-1 surface antigen on Nulli-SCC1 and NR1 embryonal carcinoma cells**

		Number of cells scored		% positive cells		% of untreated†	
Cell line	Treatment	Anti-SSEA-1	Control serum	Anti-SSEA-1	Control serum	Uncorrected	Corrected
Nulli-SCC1	none	2351	1424	36·0	10·0		
	retinol	2411	311	17·4	2·9	48·3	55·6
	retinoic acid	2392	503	11·9	8·7	33·1	12·3
NR1	none	1033	799	55·0	1·8		
	retinol	1869	152	36·1	2·0	65·6	64·1
	retinoic acid	1867	309	24·0	16·2	43·5	14·7

* Data are pooled from scoring of two coverslips from each of two experiments. Cells were plated at low density on coverslips in culture dishes and maintained (with two medium changes) for 7 days in the absence or presence of the indicated retinoid.

† Uncorrected values were calculated by determining the ratio of SSEA-1-positive cells following treatment with retinoid *versus* untreated cells. Corrected values were calculated by subtracting control serum values from anti-SSEA-1 serum values and then determining the ratios as for uncorrected values.

cell lines, there was a reduction by more than half in the percentage of reactive cells following exposure to retinoic acid for 7 days. Following the same interval of treatment with retinol, Nulli-SCC1 cells showed a more modest reduction in SSEA-1 display; reactivity with NR1 cells was also clearly diminished relative to untreated cells, although the decrease was not so great as for Nulli-SCC1 cells (Table 2).

NR1, Nulli-SCC1 and F9 cells were also tested for surface-associated laminin and fibronectin reactivity. Since these antigens were arrayed as networks over the monolayer, it was not possible to unequivocally score individual cells for reactivity. However, observed changes in the overall extent of fluorescence are recorded in Table 3. In the absence of effectors, there were either low levels of reactivity or no reactivity of the monolayers with anti-laminin or anti-fibronectin serum (e.g. Fig. 7A). Following treatment with retinol for 7 days, there was no evidence of increased reactivity with anti-laminin serum in any of the cultures. F9 cells showed clearly increased reactivity with anti-laminin serum following exposure with retinoic acid for as few as 5 days, whereas the results with the other lines were less obvious and variable under similar conditions.

There were increases in reactivity with anti-fibronectin serum when NR1, Nulli-SCC1 and F9 cultures were treated with retinoic acid for 5 or 7 days. There were also obvious increases in surface-associated fibronectin following treatment for 7 days with retinol; this increase was most dramatic with NR1 cells (see, e.g. Fig. 7B). When NR1 cells were treated with retinol for 5 days and then placed in medium without added retinol for 2 days (a protocol which leads to the re-appearance of a typical embryonal carcinoma morphology), fibronectin levels were judged to be reduced relative to those observed in continuously treated cultures, but still elevated relative to those of untreated cultures (not shown).

Immunofluorescence analyses with antiserum against the cytoskeletal component keratin indicated no reactivity in untreated cultures but obvious display of antigen when F9, Nulli-SCC1 or NR1 cells were treated with retinoic acid for seven days. Only F9 cells showed evidence of keratin expression following exposure to retinol for 7 days and under these conditions only 5–10% of the cells were scored as positive (as opposed to more than half the cells following treatment with retinoic acid).

Adhesion properties

In order to carry out immunofluorescence analyses, we plated our cells on gelatin-coated glass coverslips instead of gelatin-coated plastic dishes. NR1 cells adhered poorly to glass, even after gelatin treatment (Fig. 8A). The same was found to be true for Nulli-SCC1 cells (unpublished observations). It was noted, however, that in NR1 cultures treated with retinol or retinoic acid, the ability of the cells to adhere to the coverslips was markedly improved (Fig. 8B,C). It was also obvious that under these conditions many retinoic acid-treated NR1 cells possessed a non-embryonal carcinoma morphology whereas almost all of the cells exposed to retinol retained a morphology typical of embryonal carcinoma cells.

Table 3. *Summary of phenotypic properties of untreated and retinoid-treated NR1, Nulli-SCC1 and F9 embryonal carcinoma cells*

	Response to retinol			Response to retinoic acid		
Property	NR1	Nulli-SCC1	F9	NR1	Nulli-SCC1	F9
Morphology	change*	modest change	change	change	change	change
Plasminogen activator	modest increase	increase	increase†	increase	increase	increase†
SSEA-1	reduction	reduction	reduction†	large reduction	large reduction	large reduction‡
Fibronectin	large increase	increase	increase	increase	increase	increase
Laminin	no change	no change	no change	variable	variable	increase
Keratin	no change	no change	small increase	increase	large increase	large increase

* Change was reversible when retinol was removed.
† Eglitis & Sherman, 1983.
‡ Solter, Shevinsky, Knowles & Strickland, 1979.

DISCUSSION

The studies described with Nulli-SCC1 and F9 cells are consistent with earlier reports (Eglitis & Sherman, 1983) that these cells differentiate in the presence of retinol although the latter cells are more responsive than the former. Nulli-SCC1 cells secrete increased levels of plasminogen activator during continuous exposure to retinol for 7 to 14 days, but levels appear to fall thereafter. A similar acquired refractoriness is observed with F9 cells (Sherman, 1986*a*) and the cause appears to be epigenetic rather than genetic in nature (manuscript in preparation). Nevertheless, the delay in Nulli-SCC1 tumour appearance after a lengthy exposure of the cells to retinol suggests that at least some of the cells possess reduced tumourigenic potential under these circumstances.

NR1 cells undergo several changes in response to retinol: their morphology is altered, they show modest increases in secreted levels of plasminogen activator and some surface properties are modified: display of SSEA-1 is diminished, fibronectin levels are elevated and adhesiveness to the substratum (gelatin-coated glass or plastic) is strengthened. All of these criteria are consistent with the view that NR1 cells differentiate in response to retinol. Yet, the cells appear to revert to a typical EC phenotype following removal of the retinol. The reversal of

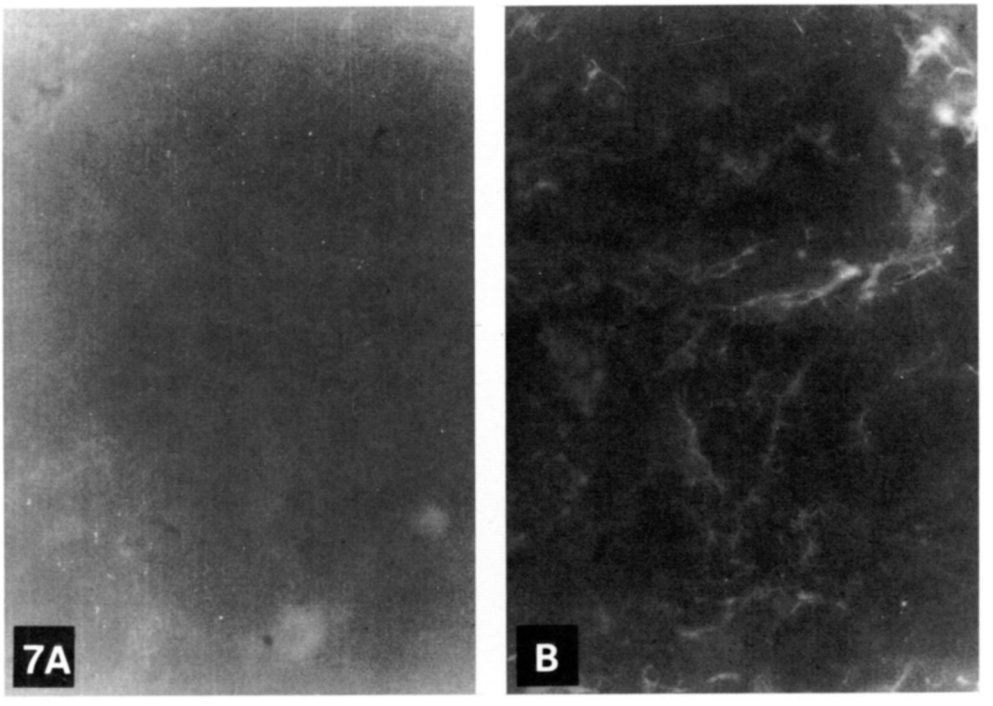

Fig. 7. Immunofluorescence analysis of cell-associated fibronectin on NR1 cells in the absence and presence of retinol. NR1 cells were cultured on gelatin-coated glass coverslips in control medium (A) or in the presence of $8{\cdot}7\times10^{-7}$ M-retinol (B) for 7 days. Coverslips were washed, fixed and assayed for fibronectin by indirect immunofluorescence. Exposure time for both figures was 60 s.

morphology is rapid and affects the vast majority of cells in the culture. This argues against the possibility that the cultures contain a small population of cells refractory to retinol which eventually overgrow the differentiated progeny

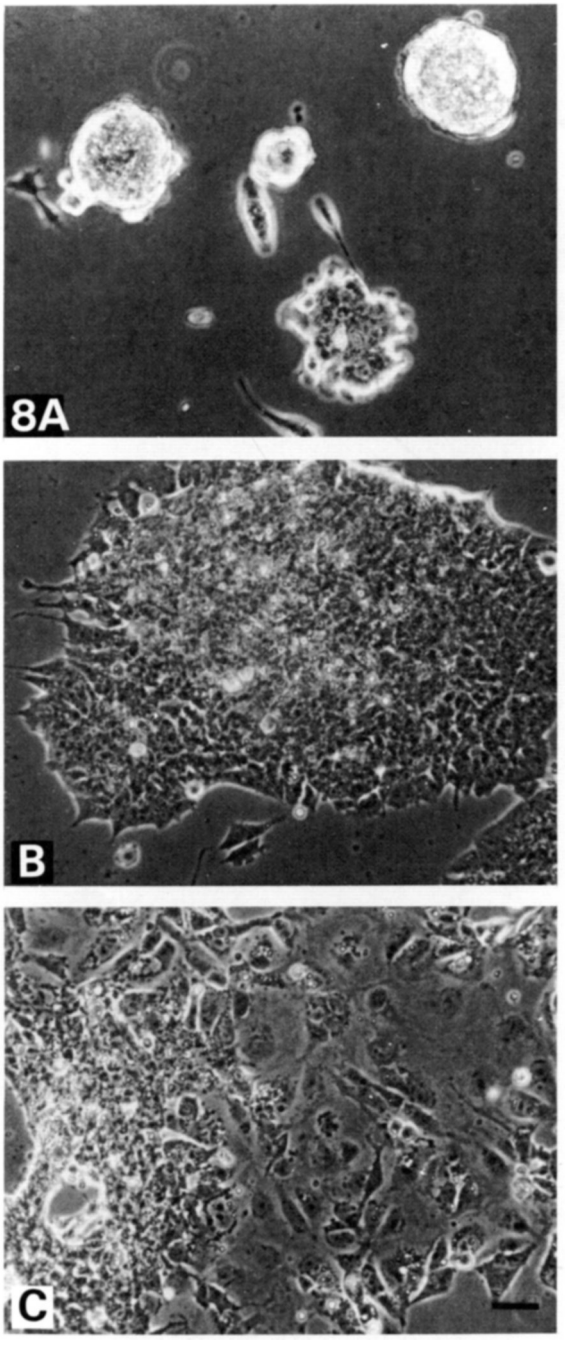

Fig. 8. Morphology of NR1 cells cultured on gelatin-coated glass coverslips in the absence and presence of retinoids. (A) Control cells after 5 days of culture; (B) cells cultured in the presence of $8{\cdot}7\times10^{-7}$ M-retinol for 5 days; (C) cells cultured in the presence of 10^{-6} M-retinoic acid for 5 days. Scale marker (in C) equals 50 μm.

following removal of the retinoid. Thus, insofar as one accepts the view that differentiation is an irreversible event, one is led to conclude that retinol fails to elicit differentiation of NR1 cells. The suggestion that even very long exposure periods to retinol are without apparent effect upon the tumourigenicity of NR1 cells supports this view since EC cells induced to differentiate *in vitro* commonly exhibit reduced tumourigenic potential (e.g. Sherman, Matthaei & Schindler, 1981). At present, we have no information about the nature of the mutation(s) in NR1 cells which influenced their response to retinol.

If NR1 cells do not undergo permanent phenotypic change in response to retinol, what is the significance of the several reversible alterations which are observed? Two likely possibilities are that (a) NR1 cells fail to progress beyond a precommitment stage of differentiation in response to retinol or (b) retinol triggers a series of changes in NR1 cells which are not directly related to differentiation even though they might commonly accompany retinoid-induced differentiation of other EC cells. The former possibility is consistent with the view expressed by Johnson, Handyside & Braude (1977) that differentiation in early mammalian embryogenesis proceeds as a series of events beginning prior to cell commitment and that such commitment is attained only after the cascade of molecular reactions reaches an irreversible stage. Insofar as the loss of ability to grow at clonal density reflects commitment of EC cells (see Sherman, 1986*a*), the studies of Rayner & Graham (1982), Rodrigues, Balicki, Newrock & Muckherjee (1985) and Sherman *et al.* (1985) suggest that most cells in PC13, F9 and Nulli-SCC1 cultures, respectively, become committed to differentiate in response to retinoic acid within 24 h, with at least some cells appearing to be irreversibly affected within 6 h. Therefore, the precommitment period when these EC cells are treated with RA is relatively short. On the other hand, Ogiso, Kume, Nishimune & Matsushiro (1982) have claimed that cells from EC line 311 pass through a 48 h reversible phase of phenotypic change in response to retinoic acid since longer exposures were required before observed alterations in surface properties were retained following removal of the retinoid. In our studies, we have found evidence of reversibility of phenotype even after several days of exposure of NR1 cells to retinol. Since NR1 cells had been exposed to mutagen, it might be argued that they are genetically blocked from progressing beyond a precommitment stage of differentiation. However, this is not a universal property of these cells since they appear to undergo characteristic and irreversible phenotypic changes in response to retinoic acid.

There is substantial precedence for retinoid-induced alterations in phenotype and growth properties of non-EC cells that appear to be independent of differentiation (see Lotan, 1981 and Sherman, 1986*b* for reviews). Most pertinent to these studies are observations that cells treated with retinoids secrete modestly elevated levels of plasminogen activator (Schroder, Chou & Black, 1980), display increased amounts of surface-associated fibronectin (Jetten, Jetten, Shapiro & Poon, 1979; Bolmer & Wolf, 1982; Keeble & Maden, 1984) and become more adherent to the substratum (Jetten *et al.* 1979) without evidence of alteration of

their state of differentiation. NR1 cells might be undergoing the same kind of changes even though their ability to differentiate in response to retinol has been extinguished. Such observations might imply that retinoids can independently influence the expression of more than one set of genes, not all of which lead to irreversible phenotypic change. However, such a conclusion would be premature for NR1 cells since it remains to be demonstrated that any of the retinol-induced alterations are dependent upon changes in gene expression. In fact, in the studies of Bolmer & Wolf (1982) retinoid-induced increases of cell-surface-associated fibronectin did not require elevated levels of transcription since this change was observed following enucleation. It is therefore conceivable that retinoid-induced changes which result in differentiation require alterations in gene expression (perhaps mediated by retinoid-binding proteins, which can translocate their ligands to the nuclear compartment; reviewed by Sherman, 1986*b*), whereas a series of reversible changes in cell appearance and behaviour can result from retinoid modifications of membrane properties, possibly through direct interaction with membrane components (see, e.g. Jetten, DeLuca & Meeks, 1982). The latter action could readily explain retinol alteration of the adhesiveness of NR1 cells to the substratum and to each other. This, in turn, could influence cell morphology as well as the antigenicity of surface-associated components.

There are long-standing arguments in the literature as to the site of action of retinoids (see Sherman, 1986*b*). If the preceding proposal is correct, then the pleiotropic effects of retinoids could result from different types of action at different cellular locations. How such retinoid actions relate to their physiological effects remains to be established. For example, it is possible that under non-pharmacological circumstances retinoids might only be presented to cells complexed to a plasma retinol-binding protein and not as free ligand (see Sherman, 1986*b*). Thus, retinoid interactions with membranes *in vivo* and *in vitro* (where retinoid levels might be substantially in molar excess of plasma retinol-binding protein) might be very different. We are currently evaluating the influence of dietary retinol and retinoic acid on differentiation of cells from several EC lines *in vivo*.

REFERENCES

BOLMER, S. D. & WOLF, G. (1982). Retinoids and phorbol esters alter release of fibronectin from enucleated cells. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6541–6545.

EGLITIS, M. A. & SHERMAN, M. I. (1983). Murine embryonal carcinoma cells differentiate *in vitro* in response to retinol. *Expl Cell Res.* **146**, 289–296.

JETTEN, A. M. (1986). Induction of differentiation of embryonal carcinoma cells by retinoids. In *Retinoids and Cell Differentiation* (ed. M. I. Sherman). Boca Raton: CRC Press (in press).

JETTEN, A. M. & JETTEN, M. E. R. (1979). Possible role of retinoic acid binding protein in retinoid stimulation of embryonal carcinoma cell differentiation. *Nature, Lond.* **278**, 180–182.

JETTEN, A. M., JETTEN, M. E. R., SHAPIRO, S. S. & POON, J. P. (1979). Characterization of the action of retinoids on mouse fibroblast cell lines. *Expl Cell Res.* **119**, 289–299 (1979).

JETTEN, A. M., DELUCA, L. M. & MEEKS, R. G. (1982). Enhancement in apparent membrane microviscosity during differentiation of embryonal carcinoma cells induced by retinoids. *Expl Cell Res.* **138**, 494–498.

JETTEN, A. M. & DELUCA, L. M. (1983). Induction of differentiation of embryonal carcinoma cells by retinol: Possible mechanisms. *Biochem. Biophys. Res. Commun.* **114**, 593–599.

JOHNSON, M. H., HANDYSIDE, A. H. & BRAUDE, P. R. (1977). Control mechanisms in early mammalian development. In *Development in Mammals,* vol. 2 (ed. M. H. Johnson), pp. 67–97. Amsterdam: North-Holland.

JONES VILLENEUVE, M. V., MCBURNEY, M. W., ROGERS, K. A. & KALNINS, V. I. (1982). Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol.* **94**, 253–262.

KEEBLE, S. & MADEN, M. (1984). Studies on vitamin A-induced pattern duplication in the regenerating axolotl limb. *J. Embryol. exp. Morph.* **82 Supplement**, 173.

KNOWLES, B. B., PAN, S., SOLTER, D., LINNENBACH, A., CROCE, C. & HUEBNER, K. (1980). Expression of H-2, laminin and SV40T and TASA on differentiation of transformed murine teratocarcinoma cells. *Nature, Lond.* **288**, 615–618.

LOTAN, R. (1980). Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim. Biophys. Acta* **605**, 33–91.

MARTIN, G. R. & EVANS, M. J. (1975). The formation of embryoid bodies *in vitro* by homogeneous embryonal carcinoma cell cultures derived from isolated single cells. In *Teratomas and Differentiation* (ed. M. I. Sherman & D. Solter), pp. 169–187. New York: Academic Press.

MCCUE, P. A., MATTHAEI, K. I., TAKETO, M. & SHERMAN, M. I. (1983). Differentiation-defective mutants of mouse embryonal carcinoma cells: Response to hexamethylenebisacetamide and retinoic acid. *Devl Biol.* **96**, 416–426.

OGISO, Y., KUME, A., NISHIMUNE, Y. & MATSUSHIRO, A. (1982). Reversible and irreversible stages in the transition of cell surface marker during the differentiation of pluripotent teratocarcinoma cell induced with retinoic acid. *Expl Cell Res.* **137**, 365–372.

RAYNER, M. J. & GRAHAM, C. F. (1982). Clonal analysis of the change in growth phenotype during embryonal carcinoma cell differentiation. *J. Cell Sci.* **58**, 331–344.

RODRIGUES, M., BALICKI, D., NEWROCK, K. M. & MUCKHERJEE, B. B. (1985). Lack of correlation between loss of anchorage-independent growth and levels of transformation-specific p53 protein in retinoic acid-treated F9 embryonal carcinoma cells. *Expl Cell Res.* **156**, 22–30.

SCHINDLER, J., MATTHAEI, K. I. & SHERMAN, M. I. (1981). Isolation and characterization of mouse mutant embryonal carcinoma cells which fail to differentiate in response to retinoic acid. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1077–1080.

SCHRODER, E. W., CHOU, I.-N. & BLACK, P. H. (1980). Effects of retinoic acid on plasminogen activator and mitogenic responses of cultured mouse cells. *Cancer Res.* **40**, 3089–3094.

SHERMAN, M. I. (1986*a*). Differentiation of embryonal carcinoma cells: Commitment, reversibility and refractoriness. *Curr. Topics devl Biol.* **20**, 345–356.

SHERMAN, M. I. (1986*b*). How do retinoids promote differentiation? In *Retinoids and Cell Differentiation* (ed. M. I. Sherman). Boca Raton: CRC Press (in press).

SHERMAN, M. I., STRICKLAND, S. & REICH, E. (1976). Differentiation of early mouse embryonic and teratocarcinoma cells *in vitro*: Plasminogen activator production. *Cancer Res.* **36**, 4208–4216.

SHERMAN, M. I., MATTHAEI, K. I. & SCHINDLER, J. (1981). Studies on the mechanism of induction of embryonal carcinoma cell differentiation by retinoic acid. *Ann. N.Y. Acad. Sci.* **359**, 192–199.

SHERMAN, M. I., GUBLER, M. L., BARKAI, U., HARPER, M. I., COPPOLA, G. & YUAN, J. (1985). Role of retinoids in differentiation and growth of embryonal carcinoma cells. *Ciba Found. Symp.* **113**, 42–60.

SOLTER, D. & KNOWLES, B. B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. natn. Acad. Sci. U.S.A.* **75**, 5565–5569.

SOLTER, D., SHEVINSKY, L., KNOWLES, B. B. & STRICKLAND, S. (1979). The induction of antigenic changes in a teratocarcinoma cell line (F9) by retinoic acid. *Devl Biol.* **70**, 515–521.

STRICKLAND, S. & MAHDAVI, V. (1978). The induction of differentiation in teratocarcinoma cells by retinoic acid. *Cell* **15**, 393–403.

WOLFE, J., MAUTNER, V., HOGAN, B. & TILLY, R. (1979). Synthesis and retention of fibronectin (lets protein) by mouse teratocarcinoma cells. *Expl Cell Res.* **118**, 63–71.

ZETTER, B. R. & MARTIN, G. R. (1978). Expression of a higher molecular weight cell surface glycoprotein (lets protein) by preimplantation mouse embryos and teratocarcinoma stem cells. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2324–2328.

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